TECHNICAL MANUSCRIPT 77

HIGH IODINATION OF ANTISERUM GLOBULINS WITH I^{127}

AUGUST 1963

UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK

U.S. ARMY BIOLOGICAL LABORATORIES
Fort Detrick, Frederick, Maryland

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The work reported here was performed under Project 4B11-05-014, "Research on BW Rapid Warning Systems," Task -02, "Biological Principles of BW Detection." The expenditure order was 2018. This material was originally submitted as Manuscript 5149.

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Project 1C022301A07

August 1963

ABSTRACT

Brucella antiglobulins were highly iodinated with nonradioactive I^{127} to determine the extent to which oxidation by iodine is responsible for loss of antibody activity. As many as 51 atoms of iodine were bound to a molecule of antiglobulin without causing loss of activity. Distribution studies of the iodotyrosines formed during iodination showed that both monoiodotyrosine and diiodotyrosine increase with increased iodination levels. When approximately 24 atoms of iodine per molecule of protein have been bound, the former begins to level off while the latter continues to rise almost linearly. The two iodotyrosines are equally distributed in a sample containing about 38 atoms of iodine per molecule of protein. The binding of 51.1 atoms of iodine per molecule of protein corresponds to the iodination of about 28 out of a total of 55 tyrosyl residues. It is believed that the remaining 27 tyrosyl residues are buried within the protein molecule and become accessible only after the iodine penetrates the exposed protective groups surrounding the molecule. Ultraviolet spectrophotometry methods for rapid estimation of bound iodine atoms and the number of iodotyrosyl residues in a sample of iodinated protein are described.

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I. INTRODUCTION

The labeling of proteins with iodine has been widely used as a tool for *in vivo* localization studies and in serological-immunochemical reactions.

Immunochemical methods are unique in their specificity and sensitivity for the detection and quantitation of small quantities of biological components. The common basis for these methods is the union of antibody protein with its relevant antigen, which appears to take place by some mechanism involving selective attraction by weak interatomic forces at definite complementary areas. The labeling of antibody protein with radioactive I^{131} (half-life, eight days) has greatly increased the speed and accuracy of immunochemical analysis. Serological investigations have taken advantage of the multivalency of complex antigens (i.e., the ability of a single antigen to combine with several molecules of labeled antibody protein) in devising extremely sensitive procedures for the detection of antigen.

Above certain limits, the iodination of proteins with natural I^{127}, containing traces of I^{131}, has resulted in the destruction of antibody activity. Pressman *et al*[1] have shown progressive decreases in activity with an increase of iodination level. They were able to produce an antibody protein with 18 atoms of iodine per molecule (mol) of protein without completely destroying its activity. However, Butement,[2] Francis *et al*,[3] and Rajam *et al*[4] suggested more conservative levels of iodination. While the upper limit of iodination varied somewhat from author to author, this limit appeared to be about six to eight atoms of iodine per mol of globulin. The loss of activity was generally attributed to the oxidative action of I^{127} as well as to the self-irradiation effects of the bound I^{131}.

A study was undertaken to determine the extent to which oxidation by iodine was actually responsible for protein denaturation. Antiserum from a cow infected with *Brucella abortus* was fractionated by methanol precipitation and the bovine antiserum globulins (Ab-G) iodinated with I^{127}. The catalytic effect of iodine on the oxidation-reduction reaction in a ceric-arsenite system was used to estimate the organically bound iodine in ashed samples. Iodinated tyrosine and protein samples were subjected to simple alkaline hydrolysis without ashing and the alkali-lability of the bound iodine was ascertained. A method is suggested for the rapid estimation of protein-bound iodine by ultraviolet spectrophotometry. The antibody activity of iodinated Ab-G was determined by the antigen-antibody agglutination tests.

The investigation was extended to include the possible mechanisms involved in iodine binding by hydrolyzing the iodinated proteins enzymatically and examining the hydrolyzates for iodoamino acids by paper chromatography. An ultraviolet method is described for the rapid estimation of the iodotyrosyl residues in a molecule of protein by differential spectrophotometry.

II. EXPERIMENTAL MATERIALS AND METHODS

A. FRACTIONATION OF BOVINE ANTISERUM

Antiserum was obtained from a cow infected with B. abortus. The serum was fractionated at pH 7.8 by the cold methanol precipitation method of Dubert.[5] The protein was redissolved in 0.85 per cent NaCl, pH 8, and dialyzed at 5°C in 0.01M phosphate buffer (containing 0.85 per cent NaCl), pH 7.2, for 24 hours to eliminate traces of methanol. Samples were taken for protein analysis and paper electrophoresis. The remainder of the Ab-G was immediately stored at -20°C.

Paper electrophoresis was carried out in a horizontal migration chamber and the paper stained with bromphenol blue.[6] The stained protein components were eluted with 0.01N NaOH and read photometrically at 590 mμ. No corrections were made for the differential staining of the protein components. Protein analysis was performed by the Biuret reaction. The fractionation of the whole antiserum by methanol precipitation resulted in a product containing 95 per cent γ-globulin, 4.5 per cent β-globulin, and 0.5 per cent α-globulin. The whole antiserum, serving as a control to delineate the separated fractions of the antiserum globulins, showed 41.5 per cent γ-globulin, 11 per cent β-globulin, and 13.6 per cent α-globulin. Approximately 90 per cent of the original γ-globulin was recovered in the fractionated serum.

All calculations given in the text are based on a molecular weight of 160,000 for the Ab-G.

B. IODINATION OF Ab-G

A convenient method for preparing iodine solutions is the reaction of sodium iodide with potassium iodate in an acid solution. Stock solutions of the reagents are easily prepared and are stable. In the reaction, five-sixths of the liberated iodine is derived from the iodide ion and only one-sixth from the iodate ion. The use of potassium iodate is preferred to persulfate[7] as an oxidizing agent because the latter tends to produce undesirable side effects on the protein with concomitant loss of antibody activity (unpublished data). The stoichiometric proportions of iodate employed result in its complete reduction to iodine. Because of the relative insolubility of iodine in water, excess sodium iodide is used to dissolve the iodine as the triiodide.

In a typical iodination in which it is desired to iodinate 100 milligrams of protein with 60 atoms of iodine per mol of Ab-G, the following procedure is employed: (a) prepare 100 milligrams of protein in 4.0 milliliters of a 0.2M carbonate buffer, pH 9.3; (b) prepare the iodine solution

by mixing 1.2 milliliters sodium iodide (32 milligrams per milliliter), 0.3 milliliter potassium iodate (18 milligrams per milliliter), 0.45 milliliter 1.0N HCl, and 2.05 milliliters distilled water. To the protein solution, add 0.075 milliliter of 1.0M sodium carbonate and mix. Immediately add 1.0 milliliter of the iodine solution, drop by drop, with gentle agitation. Allow the iodination to proceed for ten minutes. Add 0.45 milliliter of sodium bisulfite (4 milligrams per milliliter) and mix to stop the reaction by reducing unreacted iodine. Dialyze, with one change of buffer, for 48 hours at 5°C in three liters of 0.01M phosphate buffer (containing 0.85 per cent NaCl), pH 7.8. One to two drops of toluene is added to the buffer as a preservative.

Iodination levels may be controlled by a suitable variation in the amounts of NaI, KIO_3, and HCl used. For example, doubling the amounts of these reagents and diluting to 4.0 milliliters with water will provide sufficient iodine to introduce 120 atoms of iodine per mol of protein in the foregoing procedure. The amounts of 1.0M sodium carbonate and sodium bisulfite subsequently added must also be doubled. For higher iodination levels, more concentrated reagents must be used. Iodination levels greater than 100 atoms of iodine per mol of protein require ice-bath temperature to minimize loss of antibody activity.

If desired, the protein content of samples may be increased, but too high a concentration of protein may lead to difficulties by increasing the viscosity of the solution and by exposing the protein to excessive amounts of iodine in a confined area at a particular instant with resultant denaturation. The optimum pH of the iodination reaction is 9.3 and should not be allowed to exceed 9.5 because of possible oxidation of the iodine to iodate.[8] Dialysis of iodinated proteins at pH 7.2 results in some precipitation of protein, the extent of precipitation increasing with the degree of iodination. At pH 7.8, less insoluble protein material is produced. In a protein sample containing 1.3 milligrams of unbound iodide per milliliter, dialysis removes about 70 per cent of the inorganic iodide in 24 hours and about 99 per cent in 48 hours.

C. DETERMINATION OF PROTEIN-BOUND IODINE

Chemically clean 13- by 100-millimeter Pyrex test tubes are used throughout the procedure. Distilled water is redistilled from an all-glass still over alkali (or passed through a Deeminac or Barnstead demineralizer). Reagents are prepared from reagent-grade chemicals dissolved in the redistilled (or deionized) water. All mixing is done with a Vortex Jr. Mixer.

Treat a measured portion of one milliliter or less of the iodinated sample, containing about one milligram of protein, with three milliliters of 10 per cent trichloracetic acid to precipitate the protein. Allow to

stand ten minutes and then centrifuge. If particles of protein are found floating on top of the supernatant liquid, tap the tube slightly and recentrifuge. Discard the clear supernatant. Wash the protein precipitates three times with four milliliters of trichloracetic acid—each time, break up the clumps of protein and mix thoroughly to distribute evenly throughout the liquid. After the last decantation of the supernatant fluid, allow tubes to drain over acid-washed filter paper. Dissolve the protein precipitate in five milliliters of 2M KOH. The protein precipitation, washing with trichloracetic acid, and the final solution in KOH should be carried out without interruption. Transfer suitable samples (0.1 to 0.3 milliliter), in duplicate, to 13- by 100-millimeter Pyrex tubes and dilute to 0.3 milliliter with 2M KOH. Evaporate to dryness and ash at 600 ± 25°C for 30 minutes.[9,10] Cool and take up residues in five milliliters of redistilled water and centrifuge. Transfer suitable samples of the clear supernatant, containing 0.01 to 0.07 microgram iodide, to cuvettes and make up to 4.0 milliliters with redistilled water. Perform the colorimetric estimation at 37°C by the catalytic ceric-arsenite reaction. To each tube, add 0.5 milliliter of 0.12N sodium arsenite[9] followed by 1.0 milliliter of sulfuric acid - hydrochloric acid mixture.[10] Equilibrate at 37°C and add 1.0 milliliter of 0.012N ceric sulfate reagent.[9] Measure per cent transmittancy at zero time, after ten minutes, and again after 20 minutes.

Measurements were made on a Bausch and Lomb spectrophotometer at 420 mμ. An uniodinated sample of the same protein was carried through the entire procedure and served as a blank. Iodide standards, containing 0.02 and 0.05 microgram iodide, and a reagent blank were carried through the drying and ashing process only.

The use of trichloracetic acid as a protein precipitant results in trace losses of protein, but has the advantage of not liberating the iodine incorporated in the protein molecule. The trichloracetic ion does not interfere with the colorimetric determination.

D. DETERMINATION OF PROTEIN CONTENT

Protein samples were analyzed by a modification of the method of Gornall et al.[11] The Biuret reagent contained double the concentration of constituents recommended by those authors. One volume of the concentrated Biuret was added to two volumes of protein sample and the absorbances measured after 30 minutes at 540 mμ. The content of a standard protein (Lab-Trol, Dades Reagent, Inc., Miami, Florida) was confirmed by micro-Kjeldahl. Several iodinated samples were analyzed by the Biuret reaction and by micro-Kjeldahl. The bound iodine had no significant effect on the Biuret reaction.

Protein content was also estimated by ultraviolet spectrophotometry after precipitation and washing of the protein with 10 per cent trichloracetic acid. The sample was taken up in 2M KOH and absorbance measured at 280 mμ. The protein content of uniodinated Ab-G was analyzed by micro-Kjeldahl and was used to calculate a factor relating absorbance to protein content.

E. DETERMINATION OF ANTIBODY ACTIVITY

Agglutinating antibodies for B. abortus were determined by the test tube agglutination tests. A 1:80 dilution of the Ab-G sample was arbitrarily chosen to contain one milligram protein per milliliter. Twofold dilutions in 0.85 per cent NaCl were prepared to a final dilution of 1:1280. Equal volumes (0.5 milliliter of diluted Ab-G and B. abortus antigen (Markham Laboratories, Scientific Products, Inc.) were mixed and incubated at 37°C for 18 hours[12] and 48 hours.[13] The degree of agglutination was observed through transmitted light with a 10X magnifying lens.

F. HYDROLYSIS OF IODINATED PROTEIN AND DISTRIBUTION STUDIES OF IODOAMINO ACIDS BY PAPER CHROMATOGRAPHY

To avoid the destruction of iodoamino acids, as will occur in chemical hydrolysis, the protein samples were hydrolyzed enzymatically. Digestion was accomplished with the proteolytic enzyme, Rhozyme P11, mix 156 (Rohm and Haas Co.).

To 0.5 milliliter of the dialyzed protein sample, adjusted to pH 6.8 and containing about eight milligrams protein, were added two milliliters of a five per cent solution of Rhozyme P11 in 0.02M phosphate buffer (containing 0.85 per cent NaCl), pH 6.8. The mixture was incubated at 50°C for five hours and then at 37°C for 19 hours. The reaction was stopped by the addition of 0.5 milliliter of H_2O, 1.0 milliliter of 0.5N NaOH, and 1.0 milliliter of ten per cent $ZnSO_4$. After mixing, the sample was allowed to stand for 15 minutes to complete the precipitation of protein. The precipitated protein was removed by centrifugation and discarded. The supernatant appeared colorless or had a slightly yellow color.

The chromatograms were prepared by descending chromatography, using Whatman No. 1 paper. In preliminary work, 40 to 80 microliters of hydrolyzate and 1.5 micrograms of monoiodotyrosine and diiodotyrosine standards were spotted on the paper at three-centimeter intervals. These were developed overnight at room temperature, using 75 parts n-butyl alcohol, 10 parts acetic acid, and 15 parts water as developing solvent.[14] The completed chromatogram was dried and then sprayed with a ceric-arsenite or ninhydrin reagent. The ceric-arsenite reagent was prepared by mixing, just before use, equal volumes of five per cent w/v ceric ammonium sulfate

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(or 2.5 per cent ceric sulfate) in 3.5 N sulfuric acid and 1.3 per cent w/v of sodium arsenite solutions. The iodoamino acids appeared as white spots on a yellow background after two to five minutes, depending on amount of iodinated compounds present (sensitivity of reaction, 0.3 to 0.5 microgram of iodotyrosine). The iodinated areas tend to spread slightly with time. The ninhydrin reagent was a 0.15 per cent solution in acetone. After spraying with this reagent, the chromatogram was allowed to dry. The blue-purple colors of the amino acids were developed under an infrared lamp or were allowed to develop slowly at room temperature (sensitivity of reaction, five to nine micrograms of iodotyrosine).

For the quantitative study of the distribution of the iodoamino acids, 25 microliters of the hydrolyzates were spotted on Whatman No. 1 paper that had been previously washed with two per cent acetic acid, rinsed with redistilled water, and dried. The hydrolyzate of an uniodinated protein was included to serve as a blank. About 15 micrograms of monoiodotyrosine and diiodotyrosine were spotted at the edge of the same paper sheet. After development of the chromatogram, the strip containing the standards was cut from the sheet and stained with ninhydrin. The stained strip served as a guide for marking off the separated components. The delineated areas were excised and each cut into narrow strips for insertion into labeled, chemically clean Pyrex test tubes. The uniodinated sample, as a protein blank, was similarly marked off and excised. The narrow strips were wet with 0.2 milliliter of 2N KOH, and 3.5 milliliters of redistilled water were added. The solutions were mixed at intervals to complete the elution of the iodoamino acids from the paper. After 10 minutes, the tubes were centrifuged. Suitable portions of supernatant fluids (0.1 to 0.4 milliliter) were transferred to 13- by 100-millimeter chemically clean Pyrex tubes and 0.3 milliliter of 2N KOH was added to each tube. The mixture was then dried, ashed, and analyzed for iodide content by the ceric-arsenite catalytic reaction.

III. RESULTS AND DISCUSSION

A. ALKALI-LABILITY OF ORGANICALLY BOUND IODINE

Bowden et al[15] reported that aromatic compounds, containing one or more iodine atoms in positions ortho to electron-donating groups (e.g., -OH, $-NH_2$), readily released some of the bound iodine in an acid solution of ceric ion. Aqueous solutions of monoiodotyrosine (MIT) and diiodotyrosine (DIT) were subjected to the ceric-arsenite colorimetric reaction without prior drying or ashing. About 63 per cent of the iodine bound to diiodotyrosine and about 49 per cent of that bound to monoiodotyrosine was measurable by this reaction, evidently indicating partial liberation of the bound iodine. When aqueous solutions of the iodotyrosines in 2M KOH were evaporated to dryness at 105°C without ashing (essentially, alkaline hydrolysis) and the residues analyzed for iodide, total liberation of the bound iodine occurred. Paper chromatographic and ultraviolet spectrophotometric studies on the dried residues indicated complete destruction of MIT and DIT, including its tyrosine moiety. On the other hand, alkaline hydrolysis of tyrosine did not destroy its structural entity.

Iodinated protein samples released only about 30 per cent of the bound iodine in the direct reaction with the ceric-arsenite reagents. Alkaline hydrolysis, however, liberated 90 to 100 per cent of the protein-bound iodine. This finding, together with the high sensitivity of the ceric-arsenite reaction, suggests a rapid and specific technique for the detection of microorganisms by means of antibody globulins labeled with non-radioactive iodine.

B. ESTIMATION OF PROTEIN-BOUND IODINE BY ULTRAVIOLET SPECTROPHOTOMETRY

Ultraviolet spectrophotometry may be used as a simple and rapid means of obtaining an estimate of protein-bound iodine. Hughes and Straessle[16] showed that iodinated proteins, at pH 10.75, shifted from 279 to 312 mμ as a result of formation of the iodo-derivatives of tyrosine, and that the absorbance at 312 mμ increased progressively with the degree of iodination.

In an alkaline solution, proteins absorb significantly between 270 and 300 mμ because of chromophoric aromatic amino acids, tryptophan and tyrosine. Tryptophan shows little or no change in spectra or intensity of absorption with change of pH. On the other hand, tyrosine contains a readily ionizable phenolic group and shows a gradual shift in its maximal peak from 275 mμ at pH 7 to 293 mμ at pH 13 with an increase in intensity of absorption as the phenolic groups become more completely ionized. Slight variations in pH, in this range, would result in changes of absorbance at specified wavelengths, whereas at pH 13 or above, the effect of pH becomes much less critical. Tyrosine is the major amino acid in the protein

molecule undergoing iodination and, like tyrosine, the intensity of absorption of the iodotyrosines tends to increase with the ionization of the phenolic groups.

The absorbance of a sample at 280 mμ in a 2M KOH solution was related to its protein content ($E\ ^{1\%}_{1\ cm}$ Ab-G = 14.7). The absorbances of mono- and diiodotyrosine at this wavelength, in respect to their tyrosine moiety, are comparable to tyrosine alone. Therefore, the conversion of tyrosine to its iodo-derivative has little effect on the absorbance at 280 mμ. Calibration curves must be prepared for the particular protein under study because the number of tyrosine and tryptophan molecules in a molecule of protein varies with the type of protein. DIT absorbs maximally at 310 to 312 mμ ($E\ ^{1\%}_{1\ cm}$ = 130) where the absorbance due to protein is minimal. Since DIT (at 312 mμ) and protein (at 280 mμ) show adherence to Beer-Lambert's law of linearity, it is possible to apply the principle of multicomponent analysis to a mixture of these substances. On solving the simultaneous equations, taking into consideration the molecular weights of the two components and that there are two atoms of iodine per molecule of DIT, the final equation reduces to:

For general protein,

Atoms of iodine per mol of protein =

$$\frac{226\left(\frac{A_{Pr\ at\ 280\ m\mu}}{C_{Pr}}\right) A_{312} - 242\left(\frac{A_{Pr\ at\ 312\ m\mu}}{C_{Pr}}\right) A_{280}}{4.0\ A_{280} - 1.0\ A_{312}}$$

and for Ab-G

Atoms of iodine per mol of protein =

$$\frac{331\ A_{312} - 71\ A_{280}}{4.0\ A_{280} - 1.0\ A_{312}},$$

where A_{Pr} = absorbance of protein at specified wavelengths,

C_{Pr} = known concentration of protein having absorbance A_{Pr} (in mg/ml), and

A_{312}, A_{280} = absorbance of iodinated protein at 312 and 280 mμ, respectively.

The method may be used on trichloracetic acid - precipitated protein or directly on dialyzed samples. In the former, the protein precipitate is taken up in 2M KOH; in the latter, five volumes of 2M KOH are added to one volume of sample. The absorbance of the solution, containing 0.6 to 2.5 milligrams of protein, is measured at 280 and 312 mμ.

Inasmuch as the use of the formula assumes that all iodotyrosyl residues are in the form of diiodotyrosine, the method is largely empirical. Some comparative values by chemical assay and ultraviolet spectrophotometry are given in Table I.

TABLE I. ATOMS OF IODINE PER MOL OF PROTEIN BY CHEMICAL ASSAY AND ULTRAVIOLET SPECTROPHOTOMETRY*

CHEMICAL ASSAY, atoms I/mol protein	ULTRAVIOLET SPECTROPHOTOMETRY, atoms I/mol protein
42	40.3
51.1	48
57.3	56
61.4	57.1
66.3	61.5
11.7	11.6
23.6	24.2
33.2	35
38.8	38.8
54	51.6
21.7	21.5
40.7	43
79	80

* Protein-bound iodine, by chemical assay, was determined on the samples precipitated and washed with trichloracetic acid and taken up in five milliliters of 2M KOH. Samples were dried and ashed for colorimetric estimation of iodide content. Protein-bound iodine, by ultraviolet spectrophotometry, was estimated from absorbances of the alkaline solutions at 280 and 312 mμ. The alkaline solutions of the iodinated samples were allowed to stand at least 10 minutes before absorbances were measured in 1.0-cm cells on a Beckman Spectrophotometer, Model DU. Absorbances were substituted in the formula: $\frac{331\ A_{312} - 71\ A_{280}}{4.0\ A_{280} - 1.0\ A_{312}}$

C. EXTENT OF IODINE BINDING TO Ab-G

Dialyzed Ab-G were iodinated with levels of iodine ranging from 10 to 200 atoms of iodine per mol of protein.

The number of atoms of bound iodine per mol of protein (a.b.i.) increased as the number of atoms of iodine introduced into the protein sample was increased. This is illustrated in Table II. The per cent of introduced iodine that actually binds to the protein is shown in Column 3. Up to and including 51.1 a.b.i., there is an average recovery of 42 per cent or a loss of about 58 per cent of the iodine during the iodination process. The mechanism of iodine binding involves a study of the iodination of the amino acids comprising the protein. The amino acids that play an important role in these substitution reactions are tyrosine, histidine, and cysteine. The reactions of amino acids with iodine, in an acid medium, are well documented in the literature[8] and imply an ionic mechanism involving a cationic I^+ ion. An iodine molecule initially dissociates in solution to form one inactive anion and one active cation. This accounts for 50 per cent loss of the iodine atoms for the subsequent substitution reactions. Tyrosine adds one cation of iodine to form monoiodotyrosine (MIT) and then takes on a second cation to form diiodotyrosine (DIT). The cations substitute at the two carbons ortho to the phenolic group. Thyroxine may also form from two molecules of DIT, with a loss of an alanine side chain from one of the DIT molecules, in an oxidizing milieu of excess iodine.[17-19] Histidine adds iodine to the 2, 5 carbons of its imidazole ring. Like tyrosine, the first cation of iodine adds slowly to form monoiodohistidine and the second cation substitutes rapidly to form diiodohistidine. However, diiodohistidine may add on a third cation of iodine to one of the nitrogen atoms of the imidazole ring to form the triiodohistidine. The N-I bond is relatively weak and the iodine atom is easily released as the inactive anion in the presence of sodium bisulfite. Destruction of histidine is also likely to occur because of the sensitivity of the imidazole ring to oxidation in the presence of a slight excess of iodine. The active iodine cation displaces the hydrogen in the sulfhydryl group of cysteine to form iodocysteine which, in turn, reacts with another molecule of cysteine to form the oxidation product, cystine, with the release of the inactive iodine anion. Oxidation beyond the disulfide stage may occur even in the presence of small amounts of iodine.[18] The oxidation reactions of iodine with the conversion of active iodine to the inactive form would account for the loss of the remaining eight per cent of the cationic iodine to the system. The above reaction mechanisms are generally presumed to apply also to the iodination of protein in an alkaline medium where binding of iodine to protein occurs more readily.

TABLE II. IODINATION LEVELS AND ATOMS OF IODINE BINDING TO PROTEIN*

Iodination Level, atoms iodine/mol protein	Atoms Bound Iodine/ Mol Protein	Per Cent Utilization of Introduced Iodine
10	4.4	44
20	9.4	47
40	16.4	40
60	25.2	42
80	32.7	41
100	42.0	42
120	50.0	42
125	51.1	41
150	57.3	39
175	61.4	35
200	66.3	33

* Atoms of iodine introduced per mol of protein are related to the number of atoms of iodine bound per mol of protein (by chemical assay) and per cent of introduced iodine utilized in the substitution reaction.

In the samples higher than 51.1 a.b.i., a steady drop in recovery of introduced iodine is observed. Figure 1 is a graphical presentation of the data in which atoms of iodine per mol of protein are plotted against iodination level, in atoms of introduced iodine per mol of protein. There is a linear relationship from zero to about 50 a.b.i., at which point a break occurs. The "tapering off" effect again appears linear to 66.3 a.b.i. Evidently, the protein has undergone some changes in its molecular structure to cause this reduction in iodination rate.

D. ANTIBODY ACTIVITY OF IODINATED Ab-G

Previous experiments on low or moderately iodinated samples showed no loss of antibody activity. Table III tabulates the results on highly iodinated samples. Only the 1:640 and 1:1280 dilutions are shown.

After an 18-hour incubation, the sample containing 51.1 a.b.i. shows no loss of activity when compared with the control (uniodinated Ab-G). It is interesting to note that the activity of the sample with 42 a.b.i. shows a slightly higher titer than the control. This was also observed on a previous occasion. However, these differences in activity tend to disappear after a 48-hour incubation. The reason for this is not entirely clear, but it could be attributed to an initial increase in sedimentation rate of the protein as its molecular weight (and density) increases with iodination.

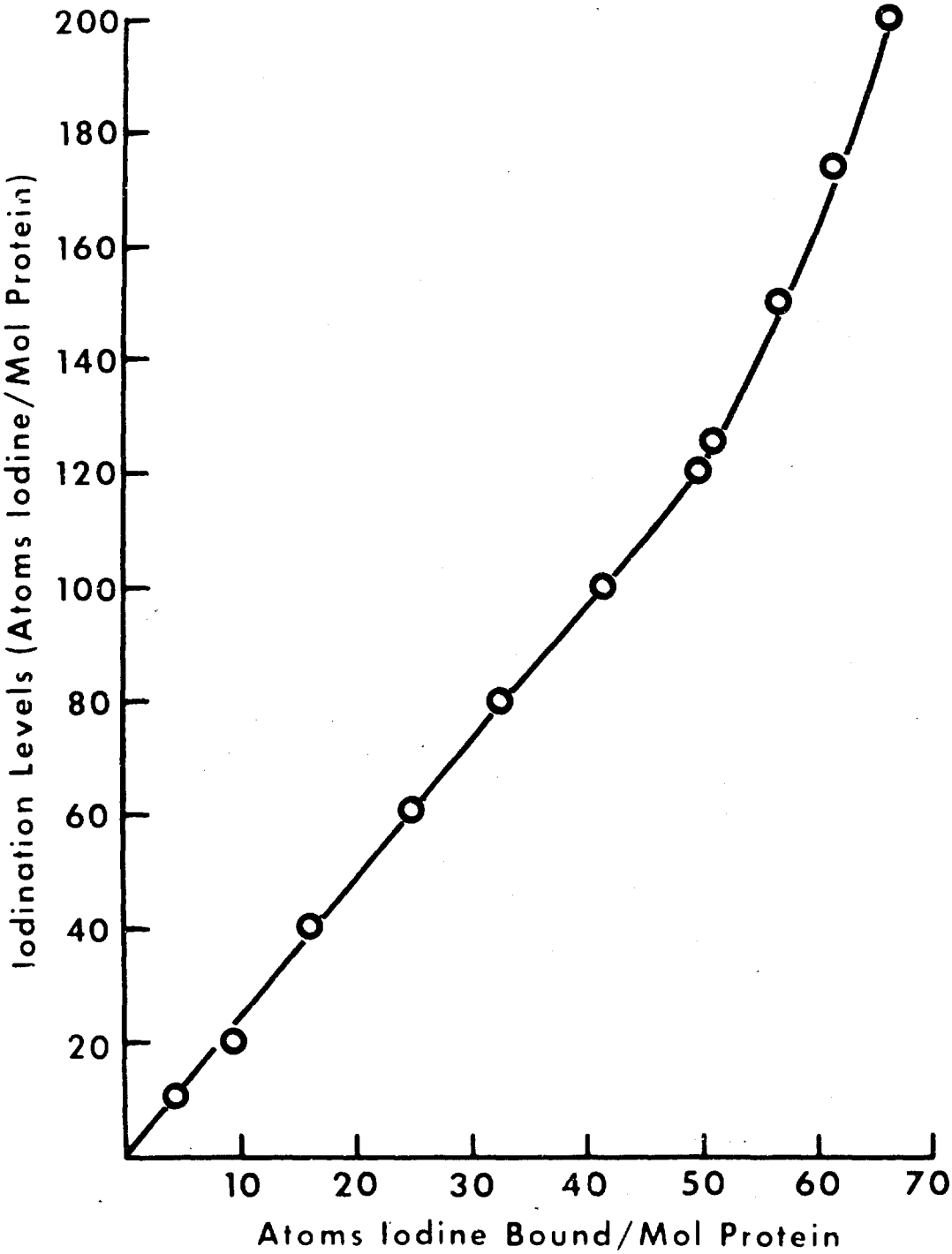

Figure 1. Atoms of Iodine Bound per Mol of Protein with Increasing Amounts of Introduced Iodine.

TABLE III. EFFECT OF HIGH IODINATION LEVELS ON ANTIBODY ACTIVITY

SAMPLE, atoms iodine/ mol protein	AGGLUTINATION TITERS			
	18-Hr Incubation Dilutions		48-Hr Incubation Dilutions	
	1:640	1:1280	1:640	1:1280
42	3+	2+	4+	4$
51.1	2+	1+	4+	4+
57.3	1+	±	3+	3+
61.4	1+	±	3+	3+
66.3	1+	±	2+	2+
0.0 (uniodinated control)	2+	1+	4+	4+

* Equal volumes of antigen and dilutions of Ab-G were incubated at 37°C for 18 hours and 48 hours. The agglutinating titers of the highest dilutions are tabulated. The 1:640 and 1:1280 dilutions contain 0.12 and 0.06 milligram of protein, respectively.

The decrease in activity beyond 51.1 a.b.i. and the concomitant break in the iodination rate curve of the protein are apparently indicative of oxidative processes that result in destruction of the protein. There do not appear to be any striking differences between antibody protein and undifferentiated globulins in their ability to bind iodine.[20,21] If simple iodine substitution reactions cause inactivation of the protein molecule, the activity should have been reduced linearly to zero with an increase in iodination. Actually, the decrease in activity began to occur in samples higher than 51.1 a.b.i., which suggests that the formation of the iodo-derivatives of the amino acids, per se, demonstrated no effect on the properties of the antibody site but that the decrease in activity was related to oxidative denaturation of the whole protein molecule.

E. DISTRIBUTION OF IODOAMINO ACIDS IN THE IODINATED Ab-G BY CHROMATOGRAPHY AND ULTRAVIOLET SPECTROPHOTOMETRY

The hydrolyzates of the iodinated samples were subjected to paper chromatography[22] and stained with ceric-arsenite reagent. The rates of migration of the separated components were compared with those obtained with reference solutions of monoiodohistidine (0.07), potassium iodide (0.18), diiodohistidine (0.36), MIT (0.48), DIT (0.60), and thyroxine (0.78). The hydrolyzates of the low-iodinated samples revealed only two

migrated areas, MIT and DIT. The more highly iodinated samples showed additional components, two of which migrated as diiodohistidine and thyroxine. An unidentified component, having a migration rate of 0.27, was also evident and is believed to be a chemically altered diiodohistidine. After two to three days, the diiodohistidine and this unknown substance disappeared altogether from the hydrolyzates and a single spot was noted in the position of inorganic iodide. No monoiodohistidine was observed on any of the samples chromatographed. This finding was surprising inasmuch as this derivative is an intermediate in the formation of diiodohistidine. It is possible that small amounts were actually present, but not in sufficient quantities to be detectable, or that the conversion of monoiodohistidine to diiodohistidine is exceedingly rapid in the presence of excess iodine.

The samples were rechromatographed on acid-washed paper. The separated components were eluted and analyzed for iodide content. The data in Table IV indicate the per cent of the protein-bound iodine that goes to form each of the iodotyrosines. Detectable diiodohistidine began to form in the sample containing 23.6 a.b.i. and increased to about five per cent of the total bound iodine in the more highly iodinated samples. Some three per cent of the bound iodine was incorporated in the unidentified iodinated substance. The per cent iodine bound as MIT decreased with an increase in iodination level and appeared to stabilizedbeyond 51.1 a.b.i. as other tyrosyl residues, heretofore shielded from attack, apparently became iodinated. Conversely, DIT increased with the iodination level as more of the MIT took on a second atom of iodine. Thyroxine formed with an increase of DIT residues and evidently reached a maximum in the sample with 61.4 a.b.i.

The data in Table IV allow one to calculate the number of tyrosyl residues converted to the iodotyrosines in each of the samples. The atoms of bound iodine multiplied by the percentages of each of the iodotyrosines gives the number of atoms of iodine that go to form each of the compounds. Since one atom of iodine binds to one molecule of tyrosine to produce MIT, the calculated number of atoms of iodine is equal to the tyrosyl residues consumed. DIT requires two atoms of iodine for each molecule of tyrosine, therefore, the calculated number of iodine atoms is divided by two. Similarly, four atoms of iodine binds to two molecules of tyrosine to form thyroxine and, again, the number of iodine atoms is divided by two.

The use of ultraviolet spectrophotometry for estimating tyrosyl residues consumed was also investigated. Beaven *et al*[23] devised a formula for calculating the number of tyrosyl and tryptophanyl residues in a molecule of protein by measuring absorbances at 280 and 294.4 mμ in a 0.1N NaOH solution. Since the iodotyrosyl residues also show significant absorption at the latter wavelength, the formula was not applicable for measuring unsubstituted tyrosyl residues. In a 1M acetic acid solution, tyrosine, DIT, MIT, and tryptophan show maximal absorption at 275, 287,

TABLE IV. DISTRIBUTION OF IODOTYROSINES IN HYDROLYZATES OF Ab-G*

SAMPLE, atoms iodine/ mol protein	Per Cent of Bound Iodine as MIT	Per Cent of Bound Iodine as DIT	Per Cent of Bound Iodine as Thyroxine
11.7	57	43	--
23.6	42	46	4
42.0	24	62	6
51.1	17	64	11
57.3	13	67	12
61.4	13	66	13
66.3	14	69	9

* Hydrolyzed iodinated samples were subjected to paper chromatography. The bound iodine was determined from areas eluted from chromtograms and per cent of total bound iodine was calculated for each of the components. Diiodohistidine and degraded products (not included in Table but accounted for in the calculations) averaged eight per cent of the total bound iodine.

283, and 279 mμ, respectively. In an alkaline solution, tyrosine and the iodotyrosines ionize as the phenolate ion, causing a shift of maxima to longer wavelengths. The spectrum of tryptophan is only slightly affected by change of pH because of the lack of an ionizable aromatic group. The spectral shift of tyrosine and its iodo-derivatives with change of pH led to the use of differential spectra for estimating unsubstituted tyrosyl residues in iodinated proteins.

In Figure 2 the differential spectra of standard solutions of tyrosine, tryptophan, DIT, and MIT are shown. Tyrosine and tryptophan show a maximum at 293 mμ, tryptophan having a relatively low intensity of differential absorption. MIT and DIT show maxima at 305 and 310 to 312 mμ, respectively. Figure 3 shows the differential spectra of variably iodinated protein samples. The uniodinated protein sample has a maximum at 294.4 mμ due primarily to the sum total absorption effects of tyrosine and tryptophan. As the number of atoms of bound iodine increases, there is a gradual shift of spectra in the direction of the maximum peaks of MIT and DIT. Samples containing 42 a.b.i. and 57.3 a.b.i. (not shown here) show peaks at 305 and 310 mμ, respectively. The sample containing 66.3 a.b.i. shows no further shift beyond 310 mμ. Evidently the peak at 310 mμ does not become apparent until some 62 per cent of the substituted tyrosyl residues are in the form of DIT.

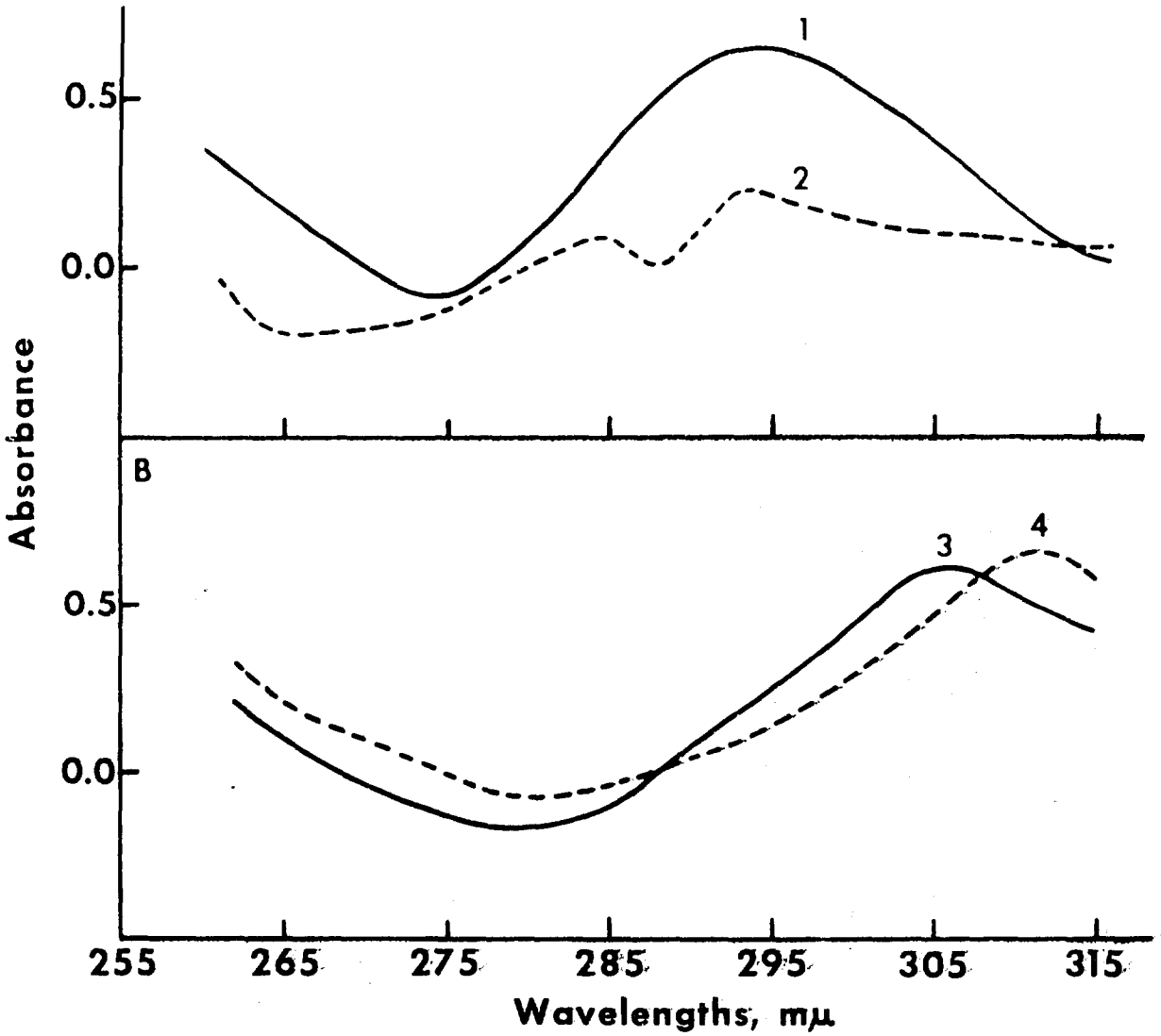

Figure 2. Differential Spectra. Stock standards were diluted 1:5 with 2M KOH and 1M acetic acid. The absorbances in acid solution were subtracted from those in alkaline solution and the differences plotted. Curve 1, Tyrosine (49.3 μg/ml), Curve 2, Tryptophan (44.5 μg/ml), Curve 3, Monoiodotyrosine (50 μg/ml), Curve 4, Diiodotyrosine (50 μg/ml).

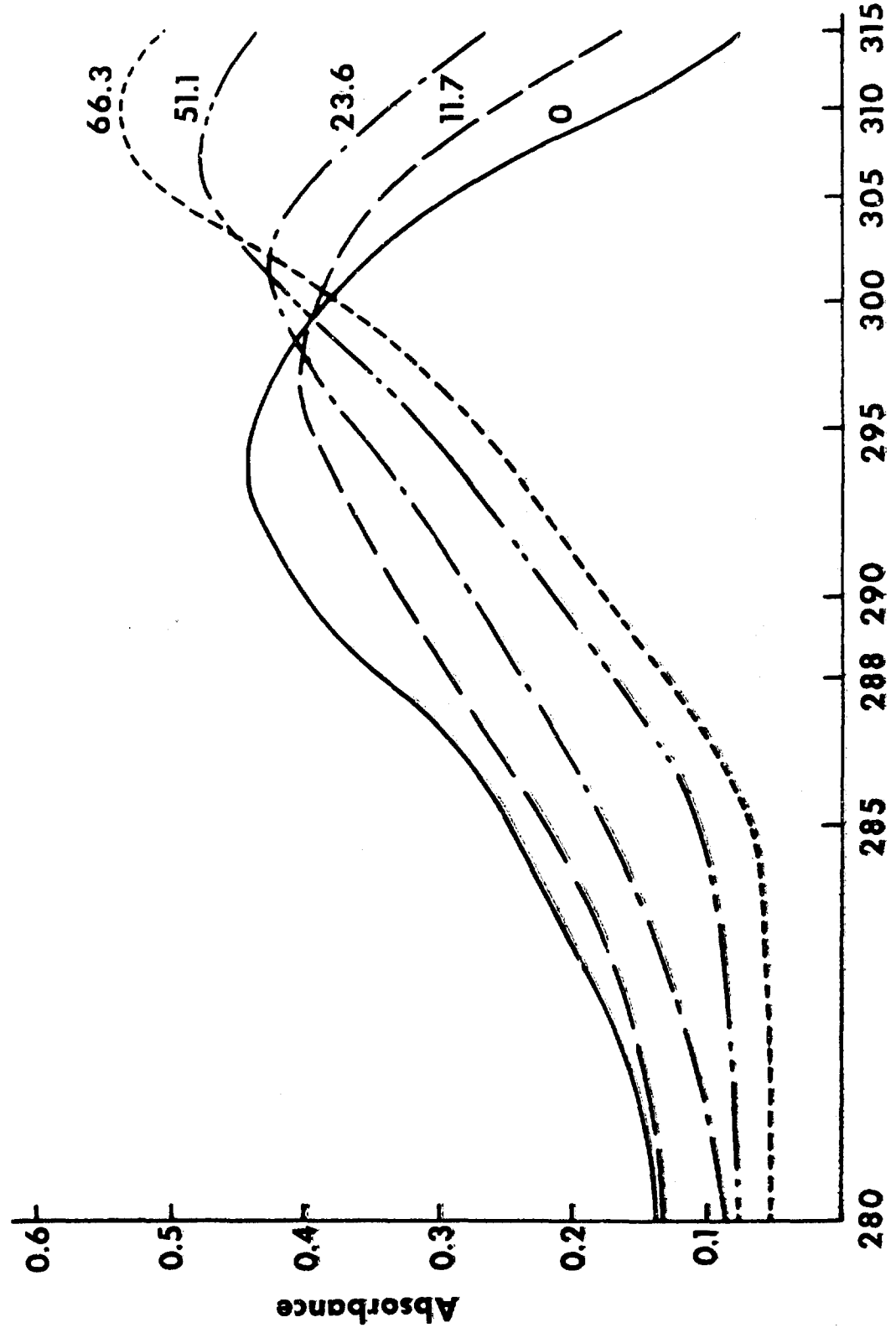

Figure 3. Differential Spectra of Iodinated Proteins. Iodinated samples and an uniodinated sample were diluted 1:6 with 2M KOH and 1M acetic acid. The absorbances in acid solution were subtracted from those in alkaline solution and the differences plotted. Numbers indicate atoms of bound iodine per mol of protein. Protein concentration, 520 micrograms per milliliter.

At 288 mμ (Figure 2), tryptophan demonstrates a minimum with negligible absorption; DIT and MIT pass through zero absorbance at or close to this wavelength. The differential absorbance of an iodinated protein at 288 mμ should essentially eliminate the effects of tryptophan and the iodotyrosines. Therefore, this wavelength was chosen for estimating the unsubstituted tyrosyl residues in a molecule of protein.

The unsubstituted tyrosyl residues can be calculated by the following formula:

$$\text{Mols unsubstituted tyrosine/mol protein} = \frac{Ax}{\text{Protein content of sample (mg/ml)}} \times \frac{\text{Conc. of tyrosine standard (mg/ml)}}{A_{std}} \times \frac{\text{M.W. of protein}}{\text{M.W. of tyrosine}}$$

Ax = Differential absorbance of sample at 288 mμ

A_{std} = Differential absorbance of tyrosine standard of known concentration at 288 mμ ($E^{1\%}_{1\ cm} = 104.5$)

Substituting and consolidating:

$$\text{Mols unsubstituted tyrosine/mol protein} = \frac{82\ Ax}{\text{Protein content of sample (mg/ml)}}$$

The uniodinated Ab-G contained 55 tyrosyl residues per mol of protein by this method. Subtracting the mols of unsubstituted tyrosyl residues per mol of iodinated protein from 55 gives the mols of tyrosine consumed to form the iodotyrosines.

In Table V, the data obtained by ultraviolet spectrophotometry are compared with those obtained by chromatography distribution. There is satisfactory agreement between the two methods. Because of the insolubility of thyroxine in acid solution, no attempt was made to correct for the small amounts of thyroxine by the ultraviolet differential method.

The mols of tyrosine consumed for each of the components are also equal to the mols of iodotyrosine produced except for thyroxine, which is divided by two because there are two mols of tyrosine for each mol of thyroxine. From the chromatographic data, the distribution of the iodotyrosines in mols per mol of protein for the several iodinated samples are presented in Figure 4. MIT rises rapidly from zero to 23.6 a.b.i. and gradually levels off. DIT rises less rapidly, but continues to rise almost linearly to 66.3 a.b.i. The two curves intersect at about 38 a.b.i., indicating that such an iodinated protein would contain equal concentrations of MIT and DIT.

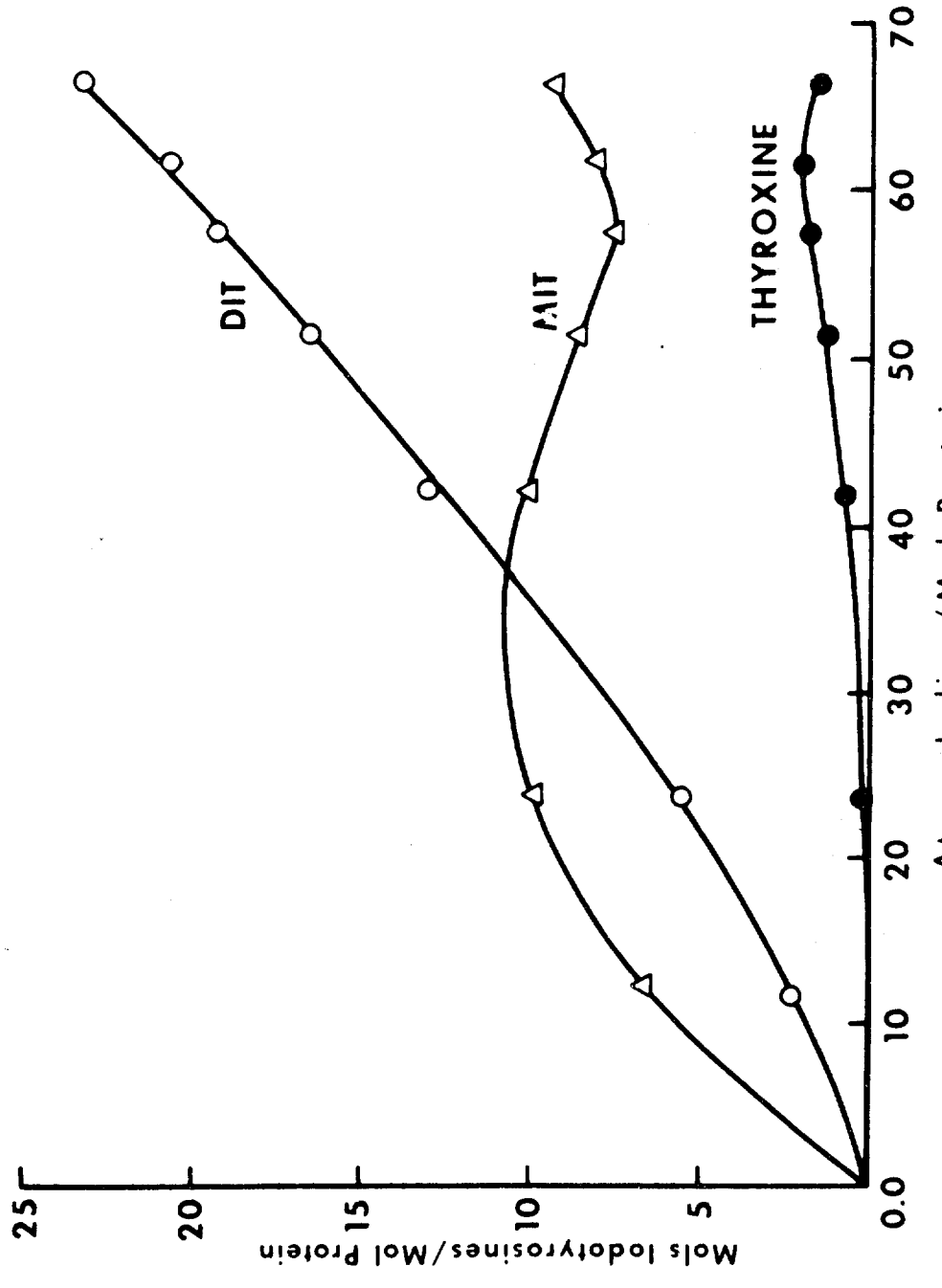

Figure 4. Distribution of Iodotyrosines (for experimental conditions, see Table IV). Mols of iodotyrosines were calculated from the per cent of total iodine bound to each of the components.

TABLE V. MOLS OF TYROSINE CONSUMED TO PRODUCE THE IODOTYROSINES*

SAMPLE, atoms iodine/ mol protein	MOLS TYROSINE CONSUMED	
	From Chromatographic Distribution	From Ultraviolet Spectrophotometry
11.7	8.7	7.5
23.6	15.7	13.0
42.0	24.3	21.9
51.1	27.8	27.4
57.3	30.2	29.0
61.4	32.3	30.0
66.3	35.4	34.1

* Values for chromatographic distribution were calculated as described in the text. For ultraviolet spectrophotometry by differential analysis, one volume of dialyzed iodinated sample, containing 0.6 to 2.5 milligrams of protein, was diluted with five volumes of 2M KOH. A similar dilution was made in 1M acetic acid. The absorbance in acid solution at 288 mμ was subtracted from the absorbance in alkaline solution at the same wavelength and the difference substituted in the formula: $\frac{85Ax}{\text{Protein content (mg/ml)}}$. The number of unsubstituted tyrosyl residues obtained is subtracted from the 55 tyrosyl residues found in the uniodinated samples to give the mols of tyrosine consumed per mol of protein to produce the iodotyrosines.

The intensely linked chains of amino acids comprising the globulin molecule are held together strongly by various attractive forces that give the molecule a three-dimensional globular structure. The action of iodine on the protein molecule would be expected to have its initial effect on those residues and linkages that are located at the surface of the globulin. The "tapering off" effect on the rate of iodination beyond 50 a.b.i. may be the result of having saturated the exposed tyrosyl residues with iodine. The concomitant decrease in antibody activity evidently signals the oxidative destruction of those chemical groups and linkages that are important in maintaining the native structure of the antibody site. The antibody molecule is known to have the same physico-chemical properties as normal undifferentiated globulins and the antibody site occupies a relatively small portion of the molecule.[24] Therefore, it is reasonable to assume that oxidative destruction of the antibody site is

associated with the destruction of the whole molecule. The continued iodination of tyrosyl residues beyond the point of the incipient denaturation of the protein is probably the result of iodine penetration through the exposed covalent linkages of the molecule to the tyrosine molecules buried within the globular mass. About 28 molecules of tyrosine in the globulin molecule were shown to be converted to the iodotyrosines in the sample containing 51.1 a.b.i. It is suggested that these 28 tyrosyl residues are readily accessible to iodination and the remaining 27 tyrosine molecules are relatively inaccessible.

The presence of MIT at the stage when all the exposed tyrosyl residues are supposedly saturated with iodine is contrary to the findings of Li,[25] who had shown that the substitution of a second atom of iodine proceeds at a faster rate than the formation of new MIT. This anomaly may possibly be explained by steric hindrance in which the remaining carbon ortho to the phenolic group is blocked by large and bulky chemical groups surrounding these MIT molecules.

After this manuscript was completed, the iodination of antithyroglobulin with I^{127} was reported in the literature.[28] The authors found that the molecular organization of the antithyroglobulin molecule was not significantly affected when iodinated at room temperature with less than 100 atoms of iodine per mol of protein, but was markedly affected when iodinated with 200 and 300 atoms of iodine per mol of protein.

IV. SUMMARY AND CONCLUSIONS

Some of the more important aspects of this investigation are reviewed here.

Apparently 51.1 atoms of nonradioactive I^{127} may be bound to a molecule of bovine antiserum globulin against Brucella abortus without loss of antibody activity. Since it has been reported that moderate iodination of proteins with I^{127}, containing traces of radioactive I^{131}, resulted in loss of activity, the I^{131} rather than the I^{127} is evidently the destructive agent.

In distribution studies of the iodotyrosines formed during iodination, it was found that both monoiodotyrosine and diiodotyrosine increase with increased iodination levels. Initially, monoiodotyrosine rises rapidly and diiodotyrosine more slowly. When approximately 24 atoms of iodine per molecule of protein have been bound, the former begins to level off while the latter continues to rise almost linearly. The two iodotyrosines are equally distributed in a sample containing about 38 atoms of iodine per molecule of protein. The binding of 51.1 atoms of I^{127} to a molecule of bovine antiserum globulin corresponds to the iodination of 28 out of a total of 55 tyrosyl residues in the protein molecule. Since further iodination results in denaturation of the protein, it is believed that the remaining 27 tyrosyl residues are buried within the protein molecule and become accessible only after penetration of the iodine through the exposed protective groups surrounding the molecule.

The presence of iodine on the tyrosyl or histidyl residues does not appear to account for loss of antibody activity. But the presence of excessive amounts of unreacted iodine tends to denature the protein by oxidation. Paper chromatographic studies have shown that demonstrable quantities of iodohistidine or thyroxine are not formed until about 30 per cent of the tyrosyl residues have been converted to monoiodotyrosine and diiodotyrosine.

The differential spectra of iodinated protein samples begin to demonstrate a peak at 310 mμ, the maximum of diiodotyrosine, when approximately 62 per cent of the iodotyrosyl residues are in the form of the diiodo-derivative.

Ultraviolet spectrophotometry may serve as a useful tool for rapidly estimating the protein-bound iodine and the number of iodotyrosyl residues in a sample of iodinated protein.

Antiserum globulins labelled with relatively high quantities of nonradioactive I^{127} have potentialities as sensitive reagents in immunochemical and serological studies. Because of the sensitivity of the chemical assay of I^{127}, as little as 0.30 microgram of iodinated antibody protein in an antibody-antigen complex can be detected when the sample of antiserum globulins contains 40 atoms of I^{127} per mol of protein. The labeled protein reagent is stable and no expensive equipment is required for analysis.

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